Elevated Oxidative Stress and Reciprocal Reduction of Vascular Endothelial Growth Factor Levels With Severity of COPD*

Hiroshi Kanazawa, MD, PhD; and Junichi Yoshikawa, MD, PhD

Study objectives: The prevalent theory concerning the pathogenesis of COPD is that it is an imbalance between oxidants and antioxidants. However, it has been reported that a decrease in vascular endothelial growth factor (VEGF) might affect the pathogenesis of COPD. Therefore, this study was designed to examine the differences between oxidative stress and VEGF levels, and the severity of COPD.

Design: Controlled cross-sectional analysis.

Setting: University hospital.

Participants: Twelve healthy control subjects and 57 COPD patients were included in this study. These COPD patients were divided into four groups based on the Global Initiative for Chronic Obstructive Lung Disease classification (mild COPD, 14 patients; moderate COPD, 15 patients; severe COPD, 16 patients; very severe COPD, 12 patients).

Measurements and results: Inflammatory markers, degree of oxidative stress, and VEGF levels were examined in sputum samples from all subjects. Nitrogen oxide levels in induced sputum were significantly higher in COPD patients than in healthy control subjects, and they increased with increases in the severity of COPD. In contrast, peroxynitrite inhibitory activity decreased with increases in the severity of COPD. Therefore, the mean (SD) peroxynitrite stress (ie, the nitrogen oxide level/peroxynitrite inhibitory activity ratio) steeply increased with increases in the severity of COPD (mild COPD: 8.4; SD, 1.5; p = 0.02; moderate COPD: 10.8; SD, 1.4; p < 0.0001; severe COPD: 14.5; SD, 2.5; p < 0.0001; very severe COPD: 18.3; SD, 4.1; p < 0.0001) compared with that of healthy control subjects. VEGF levels in induced sputum reciprocally decreased with increases in the severity of COPD (mild COPD: 1,360 pg/mL; SD, 800 pg/mL; p = 0.97; moderate COPD: 1,180 pg/mL; SD, 760 pg/mL; p = 0.50; severe COPD: 650 pg/mL; SD, 450 pg/mL; p = 0.007; very severe COPD: 480 pg/mL, SD, 240 pg/mL; p = 0.002). In addition, peroxynitrite inhibitory activity in COPD patients exhibited an accelerated decline from the mean VEGF level in healthy control subjects.

Conclusions: Elevated oxidative stress levels and a reciprocal reduction of VEGF levels in induced sputum were prominent with increases in the severity of COPD. Thus, epithelial cell injury mediated by oxidative stress may induce the decrease in lung VEGF levels, resulting in the promotion of the development of COPD.

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Key words: COPD; interleukin-8; nitrogen oxides; peroxynitrite; vascular endothelial growth factor

Abbreviations: IL = interleukin; NO = nitric oxide; PBS = phosphate-buffered saline; VEGF = vascular endothelial growth factor

COPD is a major worldwide health problem that has an increasing prevalence and mortality. Increased oxidative stress, which can be defined as an increased exposure to oxidants and decreased antioxidant capacities, is widely recognized as a central feature of COPD. Endogenous oxidants are produced by the reaction of local free radicals and intracellular reactive oxygen species, and often lead...
to extensive cellular injury and damage. Therefore, the formation of oxidants in pulmonary epithelial and inflammatory cells plays an important role in the pathophysiology of COPD. A significant increase in the expression of inducible nitric oxide (NO) synthase in neutrophils and macrophages has been observed in COPD patients, indicating that NO hyperproduction occurs in COPD patients. In fact, previous studies have reported that NO levels in adult sputum were higher in COPD patients than in control subjects. Moreover, in the lungs of COPD patients there is an increased number of neutrophils, which produce numerous superoxide anions. Thus, a simultaneous higher production of superoxide anion and NO may occur in COPD patients, potentially leading to the hyperproduction of peroxynitrite. A previous report has shown that the average rate of peroxynitrite formation could be 0.8 μmol/L/min within the whole lung and 1 mmol/L/min in the epithelial lining fluid. Most cytotoxic effects from the occurrence of high levels of NO are mediated by peroxynitrite. The overproduction of peroxynitrite, an extremely powerful oxidant, causes oxidative damage to proteins, lipids, DNA, and carbohydrates. One of the major protein modifications induced by peroxynitrite is the nitration of tyrosine residues on a variety of proteins to form the stable product 3-nitrotyrosine that may lead to a change of protein or enzyme function. Thus, exposure to peroxynitrite results in injury to or the death of epithelial cells, may be directly damaging to lung tissues, and possibly results in the development of COPD. Although it is highly reactive, its moderate rate of decomposition under physiologic conditions allows peroxynitrite to diffuse for up to several cell diameters to critical cellular targets before becoming protonated and decomposing with a nearly 30% yield to nitrogen dioxide and hydroxyl radical-like species, whereas the remaining peroxynitrite isomerizes into nitrates. Moreover, the half-life of peroxynitrite has been reported to be only 1 s at pH 7.4 and 37°C. On the other hand, we found that peroxynitrite inhibitory activity in induced sputum was reduced in patients with COPD. These findings suggest an important role for enhanced peroxynitrite stress in the pathogenesis of COPD.

However, an alternative hypothesis concerning the pathogenesis of COPD has been newly proposed based on the results of a recent study, in which there was progressive loss of alveolar wall structures through epithelial and endothelial cell apoptosis. Vascular endothelial growth factor (VEGF) induces endothelial cell proliferation, and withdrawal of VEGF leads to endothelial cell apoptosis. Thus, VEGF is a trophic factor that is required for the survival of endothelial cells. Indeed, previous studies also have reported that the protein levels and messenger RNA expression of VEGF and its receptor were decreased in lung tissue from patients with COPD, and that the decrease in VEGF might affect the pathogenesis of COPD. However, it is unknown whether a reduction in pulmonary endothelial cells leads to a subsequent loss of alveolar epithelial cells. If VEGF is critical for the maintenance of the alveolar compartment, and there is less alveolar compartment tissue in COPD patients, one might expect VEGF levels to be reduced, since there would be fewer distal alveolar septa that require VEGF signaling. Thus, it is still unclear whether the decrease in VEGF production is a cause or a consequence of COPD. Moreover, a previous study suggested that VEGF is mainly produced by pulmonary epithelial cells, and that a decrease in VEGF levels is related to epithelial cell injury. Therefore, this study was designed to examine the elevated oxidative stress to epithelial cells and consequent decreases in lung VEGF levels with increases in the severity of COPD.

Materials and Methods

Subjects

Twelve healthy control subjects were life-long nonsmoking volunteers who had no history of lung disease. Fifty-seven patients with COPD satisfied the Global Initiative for Chronic Obstructive Lung Disease criteria for the diagnosis and classification of disease severity. COPD patients were randomly enrolled from the respiratory outpatient clinic of our institution. We divided these COPD patients into four groups based on the Global Initiative for Chronic Obstructive Lung Disease classification (mild COPD, characterized by mild airflow limitation [FEV1/FVC < 70% predicted but > 80% predicted]; moderate COPD, characterized by the impairment of airflow limitation [FEV1 > 50% predicted but < 80% predicted]); severe COPD, characterized by the further impairment of airflow limitation [FEV1 > 30% predicted but < 50% predicted]; very severe COPD, characterized by severe airflow limitation [FEV1 < 30% predicted] or the presence of chronic respiratory failure [PaO2 < 60 mm Hg]). All COPD patients had a history of smoking (> 20 pack-years) and an irreversible airflow limitation (reversibility of < 10% predicted FEV1 after inhaling 200 μg of salbutamol). Their regular medication consisted of theophylline and an inhaled anticholinergic drug, but none had received oral or inhaled corticosteroids. All patients were clinically stable, and none had a history of respiratory infection for at least the 4-week period preceding the study. No subjects in this study were included as subjects with our previous studies, and therefore there is no overlap in the data of this study with data from our previous studies. All subjects gave their written informed consent for participation in the study, which was approved by the Ethics Committee of Osaka City University.

Sputum Induction and Processing

Spirometry was performed after the inhalation of 200 μg of salbutamol via a metered-dose inhaler. All subjects were in-
structed to wash their mouth thoroughly with water. They then inhaled a 3% saline solution at room temperature that was nebulized by an ultrasonic nebulizer (NE-U12; Omron Co; Tokyo, Japan) at maximum output. They were encouraged to cough deeply after a 3-min interval. The sputum sample, which was diluted with phosphate-buffered saline (PBS) solution containing dithiothreitol (final concentration, 1 mmol/L), was then centrifuged at 400g for 10 min, and the cell pellet was resuspended. Slides were made by using a cytopsin (Cytospin 3; Shandon; Tokyo, Japan) and were stained with May-Grunwald-Giemsa stain for differential cell counts. The results of differential cell counts of sputum samples were determined as the average of findings by at least three chest physicians obtained on separate occasions in a blind manner. All examiners counted 500 nonsquamous cells overall. Those percentages of differential cell counts were then averaged to give the final results. The supernatant was stored at −70°C for subsequent assay to determine interleukin (IL)-8, VEGF, and NO levels. IL-8 and VEGF concentrations were measured using an enzyme immunoassay kit (Amersham, UK, and R&D System, MN, respectively). Levels of NOs (nitrite and nitrate) in induced sputum were assayed colorimetrically after the Griess reaction as previously described.18 Two hundred-microliter sputum samples or the standard PBS solution was deproteinated by adding 20 μL of NaOH (1.0 mol/L, 4°C; Wako Chemical Co; Osaka, Japan) and 30 μL of ZnSO4 (1.3 mol/L, 4°C; Wako Chemical Co). Samples were mixed and were allowed to stand on ice for 15 min. After centrifugation (5 min, 4°C, 2600g), 100 μL of supernatant was mixed with 0.05 U of nitrate reductase (Sigma Chemical Co; St. Louis, MO), 20 μL of 0.2 mol/L N-tris (hydroxymethyl) methylamino ethanesulphonic acid (pH 7.0; Sigma Chemical Co), and 20 μL of 0.5 mol/L sodium formate (Wako Chemical Co). After anaerobic incubation at room temperature for 20 min, 1.0 mL of water was added to the samples, and nitrite was assayed in supernatants obtained by centrifugation (5 min at 260g). Deproteinized samples or standards (200 μL) were mixed with 20 μL of a 1% sulfanilamide solution (Sigma Chemical Co) in 15% phosphoric acid (Wako Chemical Co). After 10 min, 20 μL of 0.1% N-(1-naphthyl) ethylenediamine (Sigma Chemical Co) was added, and the absorption at 540 nm was determined. All subjects produced an adequate sample of sputum; a sample was considered to be adequate if the patient was able to expectorate at least 2 mL of sputum, and if, on differential cell counting, the slides contained <10% squamous cells.

Measurement of Peroxynitrite Inhibitory Activity

We chose to use the sol phase of sputum for the measurement of peroxynitrite inhibitory activity to avoid the potential confounding effects of dithiothreitol, as described in our previous study.19 The sol phase was obtained by ultracentrifuging the remaining portion of the sputum sample at 60,000g for 60 min at 4°C. This remaining portion was allocated and stored at −70°C for subsequent assay for peroxynitrite inhibitory activity. Working solutions of peroxynitrite (Wako Chemical Co) were prepared by dilution in 0.1N solution NaOH just before use as 10−2 mol/L solutions, and further dilutions were made in a PBS solution. The peroxynitrite concentration was determined spectrophotometrically by measuring the absorbance at 302 nm (εM = 1.670 mmol/L/cm). Peroxynitrite readily oxidizes dihydrorhodamine 123.20 A standard curve of oxidizing activity of dihydrorhodamine 123 to rhodamine was constructed by employing peroxynitrite. Peroxynitrite inhibitory activity was assayed by monitoring rhodamine formation at 500 nm in reaction mixtures containing a 200-μL sputum sample, 1.3-mL dihydrorhodamine 123 diluted with PBS solution (pH 7.4) and 500 μL of peroxynitrite for 30 min at 37°C. Peroxynitrite inhibitory activity was assayed at least in triplicate, and our data22 supported the specificity of this assay system for peroxynitrite. The reproducibility of our assay in this study was confirmed by repeat measurements in the same subjects on separate days.

Statistical Analysis

All values are presented as the mean (SD). Multiple comparisons among groups were analyzed by one-way analysis of variance followed by Bonferroni correction. The significance of correlations was evaluated by determining Spearman rank correlation coefficients. The relationship between VEGF level and peroxynitrite inhibitory activity in induced sputum was calculated by linear regression using the least squares method. A p value of <0.05 was considered to be significant.

RESULTS

The clinical characteristics in 57 COPD patients and 12 age-matched healthy control subjects are shown in Table 1. All patients with COPD had significant obstructive change in pulmonary function (postbronchodilator FEV1/FVC ratio, <70% predicted). The percentage of neutrophils and the concentration of IL-8 in induced sputum were significantly higher in COPD patients (mild COPD: neutrophils, 47.1% [SD, 5.5%], p = 0.027; IL-8, 33 ng/mL [SD, 0.8 ng/mL], p = 0.018; moderate COPD: neutrophils, 49.2% [SD, 7.7%], p = 0.003; IL-8, 4.0 ng/mL [SD, 1.3 ng/mL], p = 0.002; severe COPD: neutrophils, 56.4% [SD, 8.5%], p < 0.0001;
IL-8, 6.9 ng/mL [SD, 3.0 ng/mL], p < 0.0001; very severe COPD: neutrophils, 62.8% [SD, 8.6%], p < 0.0001; IL-8, 9.8 ng/mL [SD, 4.1 ng/mL], p < 0.0001) than in healthy control subjects (neutrophils, 40.1% [SD, 4.1%]; IL-8, 1.0 ng/mL [SD, 0.5 ng/mL]) (Fig 1). Moreover, the percentage of neutrophils was significantly correlated with IL-8 level in induced sputum samples from COPD patients (r = 0.87; p < 0.0001). We also found that the FEV1/FVC ratio was inversely correlated with the percentage of neutrophils (r = -0.54; p < 0.0001) and IL-8 level (r = -0.71; p < 0.0001) in these patients.

NO levels in induced sputum were significantly higher in COPD patients (mild COPD: 700 μmol/L [SD, 120 μmol/L], p = 0.0003; moderate COPD: 800 μmol/L [SD, 110 μmol/L], p < 0.0001; severe COPD: 910 μmol/L [SD, 110 μmol/L], p < 0.0001; very severe COPD: 980 μmol/L [SD, 120 μmol/L], p < 0.0001) than in healthy control subjects (540 μmol/L [SD, 80 μmol/L]) (Fig 2, left, A). These levels were significantly correlated with both the percentage of neutrophils (r = 0.72; p < 0.0001) and the IL-8 level (r = 0.72; p < 0.0001) in COPD patients. In contrast, peroxynitrite inhibitory activity in induced sputum was significantly lower in COPD patients (mild COPD: 83.1% [SD, 6.5%, p = 0.32; moderate COPD: 73.8% [SD, 6.3%, p < 0.0001; severe COPD: 63.4% [SD, 7.1%, p < 0.0001; very severe COPD: 55.1% [SD, 8.5%; p < 0.0001) than in healthy control subjects (86.0% [SD, 7.6%]) (Fig 2, right, B). Therefore, peroxynitrite stress (ie, NO level/peroxynitrite inhibitory activity ratio) was significantly higher in all four groups of COPD patients (mild COPD: 8.4 [SD, 1.5], p = 0.023; moderate COPD: 10.8 [SD, 1.4], p < 0.0001; severe COPD: 14.5 [SD, 2.5], p < 0.0001; very severe COPD: 18.3 [4.1], p < 0.0001) than in healthy control subjects (6.3 [SD, 1.0]). Peroxynitrite stress was inversely correlated with both FEV1 percent predicted (r = -0.82; p < 0.0001) and FEV1/FVC ratio (r = -0.81; p < 0.0001) in COPD patients.

VEGF levels in induced sputum decreased with severity of COPD (mild COPD: 1,360 pg/mL [SD, 800 pg/mL]; moderate COPD: 1,180 pg/mL [760 pg/mL]; severe COPD: 480 pg/mL [240 pg/mL]) (Fig 3). However, VEGF levels were significantly lower only in patients with severe COPD (p = 0.007) or very severe COPD (p = 0.002) than those in healthy control subjects (1,350 pg/mL [SD, 840 pg/mL]). We examined the correlation between peroxynitrite inhibitory activity and VEGF level in COPD patients. Peroxynitrite inhibitory activity in COPD patients exhibited an accelerated decline from a VEGF level of nearly 1,350 pg/mL, which is the mean VEGF level in healthy control subjects (Fig 4).

**DISCUSSION**

The novel aspect of this investigation is the finding of an increased exposure to NO even in patients with...
mild COPD. The percentage of neutrophils and IL-8 levels in induced sputum were also significantly higher in these patients than in healthy control subjects. IL-8 is a cytokine that is synthesized by a variety of inflammatory cells in the lung and is a potent activator of neutrophils. IL-8 induces superoxide anion release from neutrophils in vitro, and the IV administration of IL-8 in vivo has been reported to induce the accumulation of neutrophils in the lung. Therefore, the expression of IL-8 may be critically important in the pathogenesis of COPD. In the present study, we found that IL-8 levels increased with increases in the severity of COPD, and that these levels were associated with airflow limitation in COPD patients. Prevailing theories for the pathogenesis of COPD have focused on oxidant production from neutrophils stimulated by IL-8. Indeed, we also found that neutrophilic inflammation was associated with increased NO production in COPD patients.

Airway inflammation appears to play an important role in the pathogenesis of COPD, and it has been shown that the production of superoxide anions and NO by inflammatory cells is increased in COPD.

Figure 2. NO levels (left, A) and peroxynitrite inhibitory activity (right, B) in induced sputum in healthy control subjects and COPD patients. * = p < 0.01 vs healthy control subjects; † = p < 0.01 vs mild COPD patients; # = p < 0.01 vs moderate COPD patients; ‡ = p < 0.01 vs severe COPD.

Figure 3. VEGF levels in induced sputum in healthy control subjects and COPD patients. * = p < 0.01 vs healthy control subjects; † = p < 0.01 vs mild COPD patients; # = p < 0.01 vs moderate COPD patients.

Figure 4. Correlation between peroxynitrite inhibitory activity and VEGF level in induced sputum in COPD patients.
According to the existing evidence, it is likely that higher amounts of peroxynitrite are formed in the lung of COPD patients. However, the cellular source of peroxynitrite in COPD patients is unclear. A previous study\textsuperscript{24} found that the production of peroxynitrite was increased in airway macrophages and neutrophils in COPD patients compared with healthy control subjects. We found that NO levels in induced sputum increased with the severity of COPD, while peroxynitrite inhibitory activity reciprocally decreased. Therefore, peroxynitrite stress markedly increased with the severity of COPD. Peroxynitrite may directly induce pulmonary epithelial cell injury or death, since peroxynitrite is a highly reactive species that was initially considered to be an important mediator of cytotoxic effects.\textsuperscript{25} Moreover, a previous study\textsuperscript{26} reported that peroxynitrite oxidizes α₁-proteinase inhibitor, leaving the lung susceptible to an unopposed proteolytic attack on its extracellular matrix, and activates matrix metalloproteinases that are released from neutrophils and macrophages, which are capable of degrading all of the components of the extracellular matrix of lung parenchyma and may cause emphysematous changes. Indeed, we found that peroxynitrite stress was significantly correlated with airflow limitation in COPD patients. In the present study, peroxynitrite inhibitory activity was assayed by monitoring rhodamine formation. The oxidation of dihydrorhodamine 123 to rhodamine is mediated by peroxynitrite, but not by superoxide anion, hydrogen peroxide, or NO. Using this method, we found that peroxynitrite inhibitory activity in induced sputum decreased with the increases in the severity of COPD. It seems likely that the thin layer of epithelial lining fluid may provide antioxidant protection against peroxynitrite and may serve as a front-line defense for epithelial cells. Moreover, epithelial cells are also a principal source of the antioxidant capacities in epithelial lining fluid. Therefore, the reduction of peroxynitrite inhibitory activity may reflect the injury or death of epithelial cells. Thus, an inadequate supply of peroxynitrite inhibitory capacities would render epithelial cells vulnerable to peroxynitrite-mediated cellular injury. We speculate that the severity of COPD might progress as a result of decreases in peroxynitrite inhibitory activity. Moreover, since peroxynitrite production is increased during acute exacerbations of COPD, susceptibility to peroxynitrite in this state might markedly increase with the severity of COPD.

However, airway inflammation may not be the sole mechanism responsible for the pathogenesis of COPD.\textsuperscript{27} For example, Kasahara et al\textsuperscript{12} demonstrated that the long-term treatment of rats with a VEGF receptor-2 inhibitor led to endothelial and epithelial cell apoptosis followed by the enlargement of the air spaces but without clear signs of acute or chronic inflammation. Taken together, these findings suggest that neutrophil inflammation and accompanying peroxynitrite stress may not be solely responsible for COPD. However, we found that increased peroxynitrite stress was present even in patients with mild COPD. Therefore, we have hypothesized that the increased peroxynitrite stress is initially associated with increased apoptosis of epithelial cells even in patients with mild COPD. Although Kasahara et al\textsuperscript{12} revealed in an examination of resected COPD lung tissue that both epithelial and endothelial cells were undergoing apoptosis, the apoptotic cells were mainly epithelial cells, which in turn affected the endothelial cells via reduced production of their survival factor, VEGF.\textsuperscript{28} These findings suggest that the altered function of epithelial cells, which are the main sites of VEGF production, may account for the decreased VEGF expression and increased endothelial cell apoptosis observed in COPD. Thus, it is reasonable to speculate that the reduction in the number of endothelial cells in COPD is secondary to the loss of epithelial cells affected by increased peroxynitrite stress.

Increasing evidence points to a direct role for paracrine signaling between endothelial cells and surrounding target organ cells.\textsuperscript{29} For example, it is overwhelmingly evident that tissues regulate vascular architecture by signaling to endothelial cells through local angiogenic agents such as the family of VEGF. On the other hand, endothelial cells produce a variety of humoral factors, growth factors, and cell surface molecules to communicate with surrounding cells. In the pulmonary alveoli, epithelial and endothelial cells may also use this type of epithelial-endothelial cell crosstalk. It is still unclear, however, how the endothelial-derived factor that is normally present in endothelial cells signals to the alveolar septal epithelial cells. However, we found that a decrease in VEGF level leads to a marked decline in peroxynitrite inhibitory activity, suggesting that the loss of endothelial cells may accelerate the injury or death of epithelial cells. In fact, a previous study\textsuperscript{30} reported that the loss of VEGF further causes epithelial cell injury mediated by oxidative stress and culminates in COPD. The findings of the present study may shed new light on the pathogenesis of COPD, and our investigation is responsible for the relationship between oxidant-antioxidant imbalance and VEGF-dependent homeostasis of alveolar walls in the lungs of COPD patients.

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REFERENCES


